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Fonck, K; Scherphof, GL; Konings, AWT

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The Effect of X-irradiation on Membrane Lipids of Lymphosarcoma Cells *in vivo* and *in vitro*

K. FONCK, G.L. SCHERPHOF † and A.W.T. KONINGS*

*Laboratory of Radiopathology, University of Groningen, Bloemsingel 1, 9713 BZ Groningen
†Laboratory of Physiological Chemistry, University of Groningen, Bloemsingel 10, 9712 KZ Groningen, The Netherlands

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Membrane lipids/Radiation/Tumor

Phospholipids of the membranes of spleen lymphosarcoma cells were radioactively labelled with the radioresistant fatty acid palmitic acid (16:0) and the radiosensitive fatty acid arachidonic acid (20:4). The effect of irradiation on the specific radioactivity of the phospholipids was studied. For the *in vivo* experiments trace amounts of radioactive palmitate or arachidonate were injected intraperitoneally into lymphosarcoma-bearing mice. Incorporation of label into the lipids of the tumorous spleen cells was monitored in control animals and in mice that were whole body irradiated after injection of the label. In both groups of animals the label was detected in the blood within minutes after injection and was found to be efficiently incorporated into the phospholipids of the tumor cells. In the irradiated animals a marked transient increase in label incorporation was observed as compared to control animals. The radiation effect was observed in the lipids of the total homogenate, purified nuclei, the mitochondrial-lysosomal fraction and in the microsomal fraction. Most experiments were performed with nuclei, which are known for their high radiosensitivity. The levels of label incorporation for palmitate and arachidonate were increased to the same extents and found to be dose dependent. For a dose of 5 Gy the increased label incorporation started immediately after irradiation and lasted for a period of about 50 minutes.

The increase in label incorporation into the phospholipids was preceded by an increase in the concentration of fatty acids in the cytosol of the tumors. Our experiments point to the occurrence of a transient increase in the flux of fatty acids through the plasma membrane as a result of irradiation and suggest that under normal physiological conditions fatty acid uptake through the plasma membrane is the rate-limiting step in the incorporation of acyl groups into the phospholipids.

Experiments with isolated tumor cells also showed an increased incorporation of fatty acids into the phospholipids after irradiation. Again the incorporation patterns of both types of fatty acids (16:0) and (20:4) were very similar. A hypotonic treatment of the cells also resulted in a similar increase in fatty acid incorporation as irradiation did; the effects of hypotonic treatment and irradiation were not additive.

*To whom correspondence should be addressed.

INTRODUCTION

The mechanisms of radiation-induced cell damage are poorly understood. Except for a large number of studies on nucleic acids, little knowledge is available at present on the events taking place at the molecular level. Because cellular membranes play an important role in the functional organization of the cell, we initiated a series of studies on radiation-induced membrane damage some years ago.[1-3]

Alterations of membrane functions as caused by radiation have been investigated predominantly in erythrocytes and were recognized as permeability changes for small ions such as Na^+ and K^+.[4-7] These radiation effects were attributed to changes in the proteins as well as in the lipid part of the membrane (for recent revies see refs. 8 and 9).

Already in 1952 Mead[10] observed radiation induced peroxidation in emulsified fatty acids. Studies by Konings et al.[11] with artificial membranes (liposomes), prepared from phospholipids extracted from cellular membranes, have indicated that especially the polyunsaturated acyl groups of the phospholipids are readily susceptible to X-irradiation-induced lipid peroxidation.

Although products of lipid peroxidation as a result of irradiation have been reported,[12] we have been unable to detect malondialdehyde as a product of radiation-induced lipid peroxidation immediately after moderate doses of ionizing radiation.[13] Likewise, no decrease in polyunsaturated acyl groups could be detected *in vivo* [Konings, A.W.T. and Trieling, W.B., unpublished results]. It is conceivable that products of lipid peroxidation, such as malondialdehyde, are rapidly metabolized by the cells beyond detection. Likewise, rapid replacement of damaged acyl groups might possibly occur. If repair of radiation damage takes place at the level of acyl group renewal in the phospholipids, the turnover rate for radiosensitive acyl groups is likely to be higher than that for the radioresistant ones. We set out to investigate if such difference in turnover rates could be detected with the aid of radioactive fatty acids. The fatty acids chosen were palmitic acid and arachiodonic acid, because they represent a radioresistant and a radiosensitive fatty acid respectively.[11] Preliminary results of these investigations have been presented elsewhere in abstract form.[14]

MATERIALS AND METHODS

Animals; tumor system. Male tumor-bearing C57Bl mice of our own laboratory strain, 3 to 4 months of age, were used for the experiments. The tumor was a lymphosarcoma which was transplanted routinely once a week into healthy mice by intraperitoneal injection of 10^6 tumor cells in 0.5 ml of 0.9% NaCl. The cells were obtained from a tumorous spleen of about 800 mg. Spleen weight increased from about 100 mg at the time of inoculation to about 800 mg on the

seventh day after injection of 10^6 tumor cells. At day six the animals were used for the experiments; the spleen weight was about 600 mg at that time and the tumor cells were in log phase. Additional information on this lymphosarcoma tumor can be found in refs. 3, 15 and 16.

Preparation and administration of the tracer fatty acids. The following radioactive fatty acids were used: [1-^{14}C] palmitic acid (RCA, Amersham, England, TRA-21), [9, 10(n)-^{3}H] palmitic acid (RCA, CFB-37) and [1-^{14}C] arachidonic acid (RCA, CFA-504). The fatty acid solutions were dried in a glass vial under a stream of N_2 and 0.001 M NaOH was added in 0.9% NaCl. After solubilizing the fatty acids at 60°C, an equal volume of a warm 2% solution of bovine serum albumin (fatty acid free, <0.005%, BSA, Sigma A6003) in 0.9% NaCl was added. Each mouse received 0.25 ml of this solution by intraperitoneal injection, containing 10—20 μCi of the ^{3}H-labelled fatty acids and 0.5—1.0 μCi of the ^{14}C-labelled fatty acids.

Irradiation. Whole body irradiations were performed with a Philips-Müller MG-300 X-ray machine operated at 200 kV and 15 mA at a dose rate of about 0.6 Gy per minute at a focus sample distance (FSD) of 51 cm. The beam was filtered with 0.5 mm Cu and 0.5 mm Al; half value layer (HVL) was 1.1 mm Cu. The mice were irradiated in plastic boxes (5 or 6 animals per box) in a stream of air. The control animals were sham irradiated under exactly the same conditions. Irradiation of isolated cells was as follows. The tumor cell suspension was divided in portions of 10 ml and irradiated in Erlenmeyer flasks (25 ml). The X-ray machine was operated as described above. The focus-sample distance was 51 cm and the dose rate 0.6 Gy/min.

Treatment of tissues; subcellular fractionation. At different times after the injection of tracer fatty acids the animals were irradiated and killed by cervical dislocation. The blood was quickly withdrawn by heart puncture with a heparinized syringe. The liver and spleen were extirpated and a 10% (w/v) homogenate was made in 0.25 M sucrose, 50 mM Tris. HCl (ph = 7.4), 2.5 mM KCl and 5 mM $MgCl_2$ (= STKM buffer). For the spleens the homogenate was made in a Dounce all glass homogenizer (clearance: 0.14 mm). The livers were homogenized in a Potter-Elvehjem homogenizer (clearance: 0.2 mm).

A crude nuclear fraction was obtained from the total homogenate by centrifugation at 900 x g during 10 minutes. The pellet was taken up in STKM and pruified over 2.3 M sucrose (in TKM) at 130,000 x g_{max} during 30 minutes in a SW-27 rotor (Beckman). The mitochondrial-lysosomal fraction was separated from the postnuclear supernatant by centrifugation at 15,000 x g during 20 minutes. Microsomes were obtained by centrifugation of the post-mitochondrial supernatant at 105,000 x g during 60 minutes in a SW-40 or SW-41 rotor (Beckman). Nuclear membranes were obtained by sonicating the purified nuclei

for 1 minute (3 x 20 seconds) with a Branson ultrasonifier (50 to 60 Watt output) in 8 ml TKM at 0°C. The chromatin was solubilized by adding solid NaCl to a final concentration of 2 M. The nuclear membranes were pelleted in a SW-27 rotor at 130,000 x g_{max} for 60 minutes and washed in TKM-buffer. All subcellular fractions were suspended in STKM prior to lipid extraction.

Isolation of tumor cells for in vitro incubation. The tumor spleen, weighing about 600 mg, was extirpated, minced with scissors and gently pressed through two layers of nylon gauze with the aid of 8—10 ml balanced salt solution (BSS, consisting of 4.5 mM D-glucose, 14 mM NaCl, 45 μM $CaCl_2$, 0.88 mM $MgCl_2$, 4.9 mM KCl and 130 mM Tris.HCl, pH = 7.6). To dissociate small cell aggregates the cells in the filtrate were squeezed twice through a syringe needle. The tumor cells were purified by centrifugation (20 min. at 1000 x g) on a Lymphoprep (Nyegaard, Norway) gradient. The cells were washed in BSS twice and resuspended in RPMI 1640 medium (Flow Laboratories, Scotland), supplemented with 10% fetal calf serum (629, Gibco Biocult, Scotland). The cells were counted and adjusted to a concentration of 0.64 x 10^7 cells/ml. All handling was done at ambient temperature.

Incubation and further handling of the cells. Incorporation of fatty acids was studied by adding a 0.5 ml solution of radioactive palmitate or arachidonate as tracer substances to a 10-ml cell suspension. The fatty acids were complexed to bovine serum albumin as described above. In a volume of 0.5 ml the following tracers were added: 0.1—2.0 μCi [9, 10(n)-^{3}H]palmitic acid (CFB-37, RCA Amersham, England) or [1-^{14}C]arachidonic acid (CFA-504, RCA) either separately or simultaneously. The flasks were incubated at 37°C in a shaking water bath. At the end of the incubation the suspensions were rapidly cooled in ice. The cells were pelleted by centrifugation at 4°C at 900 x g for 5 minutes. The cell pellet was washed in saline and centrifuged again. The cell pellet was resuspended in 4 ml of saline and total lipids were extracted.

Extraction, separation and determination of lipids. Lipid extraction from the subcellular fractions was performed as described previously.[17] Two volumes of methanol and one volume of chloroform was added to 0.8 volume of the suspension in a glass-stoppered centrifuge tube. The extraction was completed according to Bligh and Dyer.[18] Separation and quantitative determination of the lipids have been described extensively in an earlier paper.[19] Specific radioactivities of the total lipids are expressed as disintegrations per second per microgram lipid (external standard and channel ratio method). Lipids were dried in the glass counting vials, 3 ml pico-Fluor-30 was added and radioactivity determined in an Isocap/300 (Searle) liquid scintillation counter. Counting of radioactivity of silica gel scrapings from thin layer chromatography plates was done directly in 5 ml pico-Fluor-30 (Packard). Double label counting (^{3}H and ^{14}C) was performed according to program 8 of the operation manual.

RESULTS

Label incorporation in blood lipids

Fig. 1A shows that after intraperitoneal injection of [^{3}H]palmitate into tumor-bearing mice the tritium label appeared in the blood lipids promptly after injection. Within one hour the appearance of radioactivity in the blood declined to a steady-state level, which was maintained for at least five hours. The fraction of the label associated with the free fatty acids decreased rapidly during the first hour after injection to a value of approximately 20% (Fig. 1B). Hereafter a slower decrease was observed. A rapid increase of label was found in the triglyceride fraction within one hour after the injection. After this period the amount of label in the triglycerides slowly diminished. The blood phospholipids became labelled after a lag period of about 15 minutes following injection. From then on a gradual increase in radioactivity content was observed in this lipid fraction, reaching values over 60% by the end of the period of investigation.

Label incorporation in tumor lipids in vivo

Fig. 2 shows the rate of incorporation of [^{14}C]palmitate and [^{14}C]arachidonate into the total lipid fractions of the lymphosarcoma cells. For both acids a plateau phase was reached after about one hour. The labels are most rapidly incorporated into the phospholipids which were shown to contain as much as 80—90% of the total label within one hour after injection (Fig. 2). The radioactivity in the triglycerides, after reaching a maximum of approximately 20%,

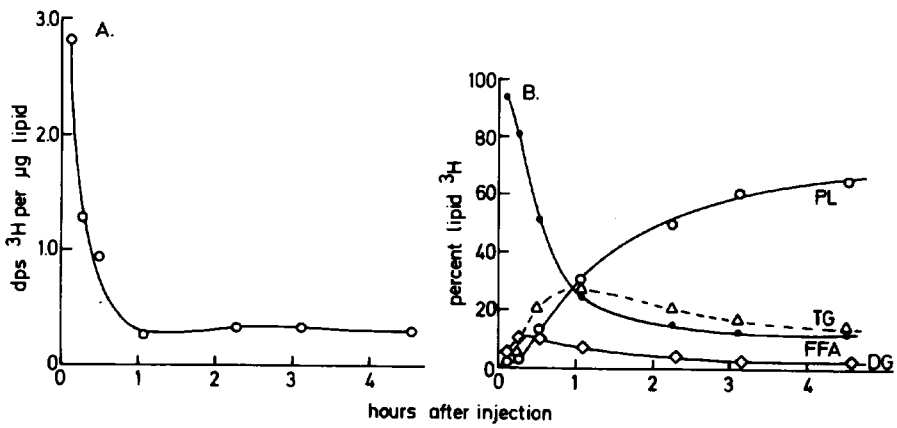

Fig. 1. Label incorporation into blood lipids after intraperitoneal injection of [^{3}H]palmitate. A Specific radioactivity of total blood lipids. B Distribution of label among the predominant labelled lipid classes. Each point represents the average of at least two experiments. PL = phospholipids; TG = triglycerides; DG = diglycerides; FFA = free fatty acids. Lipids were extracted and anlysed as described in the methods section.

started to decrease already 30 minutes after injection. A constant low percentage of the label was found in the free fatty acids of the tumor cells from one hour after injection. The incorporation patterns with [^{14}C]palmitate and with [^{14}C]-arachidonate were very similar. The same incorporation profiles as observed for total homogenates were found when purified nuclei were examined. The nuclei are of special interest because the peripheral region of the nuclei has been found to be the most radiosensitive site in the cell.[20,21] Therefore most of the results presented in this report refer to isolated nuclei.

Radiation effect on tracer incorporation in phospholipids of tumor cells in vivo

Mice were irradiated two hours after simultaneous injection of [^{3}H]palmitate and [^{14}C]arachidonate. At different intervals after irradiation the specific radioactivity of the lipids in the isolated tumor nuclei was determined. Figs. 3A and 3B show that shortly after the irradiation period the specific radioactivity of the lipids increased up to about 170 percent of the control values. The specific radioactivity dropped to control levels within 40—50 minutes after the irradiation. At a dose of 5 Gy the radiation effect starts immediately after the irradiation period and lasts for about 50 minutes.

The similarity between the effects on the two fatty acids, which becomes apparent from Figs. 3A and 3B is further emphasized in Fig. 3C which shows the isotopic ratio in the lipids. This ratio was found to remain virtually unchanged in many different experiments. The label distribution among the various lipid classes also remained unchanged after irradiation, i.e. in control as well as irradiated cells the phospholipid fraction incorporated up to 80% of the radioactivity.

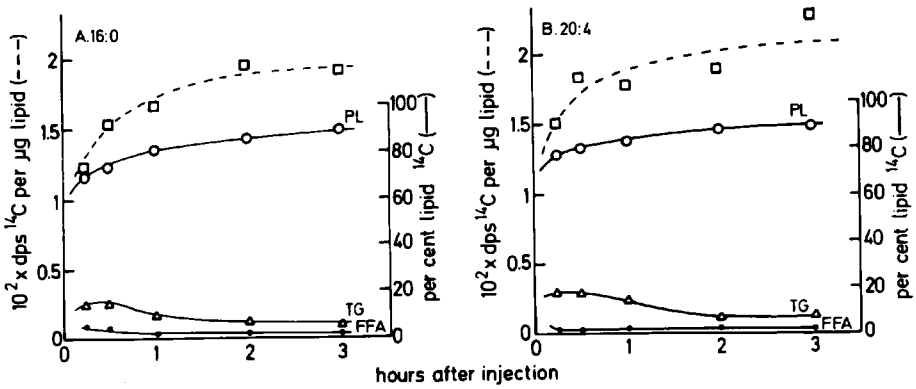

Fig. 2. Time course of labelling of the total lipid fraction of lymphosarcoma cells isolated from the tumor-bearing mice after injection of [^{14}C]palmitate (A) or [^{14}C]arachidonate (B). Each point has been obtained from two or more animals. The dashed lines represent the specific radioactivities of the total lipids; the solid lines represent the distribution of [^{14}C]label among the major lipid classes in the tumor cells.

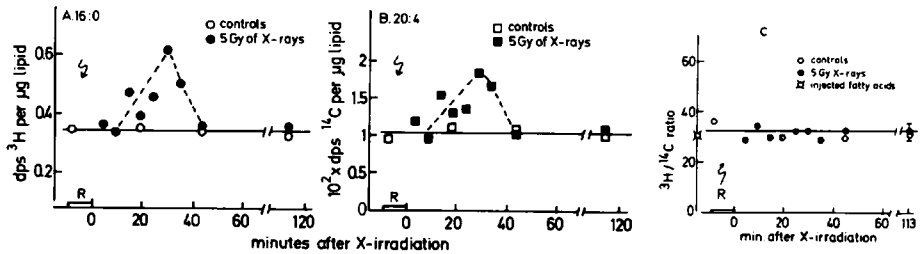

Fig. 3. The effect of X-irradiation with 5 Gy on the incorporation of simultaneously injected [^{3}H]palmitate (11 μCi) and [^{14}C]arachidonate (0.4 μCi) into nuclear lipids of tumor cells after whole-body irradiation. A Palmitic acid. B Arachidonic acid. C [^{3}H] over [^{14}C] ratios of the data from A and B. Whole-body irradiation (5 Gy) was performed two hours after label injection.

Therefore, the radioactivity per μg of total lipid can be taken as a reliable measure for the specific radioactivity of the phospholipid fraction. For reasons of convenience this was routinely done.

The radiation effect was dose dependent (Table 1), the increase in specific radioactivity being higher after 5 Gy than after 2.5 Gy. When higher doses were applied, the label incorporation started to increase already during the irradiation period. For the cause of simplicity we did not include such experiments. The increase in specific radioactivity found in whole nuclei was also observed in the lipids extracted from the nuclear membranes. The specific radioactivity increased from 0.20 dps per μg lipid in the controls to 0.30 dps per μg lipid in the irra-

Table 1. Dose-dependency of the radioation-induced increase of [^{3}H]palmitic acid incorporation into the nuclear lipids.

Tumor-bearing mice were injected with [^{3}H]palmitate, 23 μCi per mouse, and irradiated two hours after injection. The lipids of nuclei isolated from the tumor cells were assayed for radioactivity. Data are expressed as percentage of increase compared to sham irradiated controls. Two mice were used for each result.

Time after irradiation (minutes)	Increase in specific radioactivity (%)	
	After 2.5 Gy	After 5.0 Gy
11	8	10
30	20	41
42	26	45
60	14	18

diated animals 30 minutes after irradiation and 2.5 hours after injection of [^{3}H] palmitate.

The radiation effect was found to be repeatable. A second radiation dose of 5 Gy given one hour after the first dose produced a radiation effect which was virtually identical to the first one.

The radiation effect was not restricted to the nuclear lipids. It was also observed in other subcellular fractions (Fig. 4).

Availability of fatty acids as precursors for phospholipid synthesis by tumor cells in vivo.

The observations that the increased incorporation of the fatty acids was the same for palmitate and arachidonate and that the radiation response was found in all membrane fractions examined, suggest that the radiation effect represents a transient increase in the total fatty acid pool in the cell. To further explore this possibility we estimated the amounts of free fatty acid label in the soluble fraction of the cells. Two hours after injection of a tracer amount of [^{3}H]-palmitate, the mice were irradiated. The relative amount of tritium label found in the free-fatty-acid fraction started to increase shortly after irradiation and remained elevated for about one hour (Fig. 5A). In the same experiment we determined the specific radioactivity of the total lipid fraction in the isolated

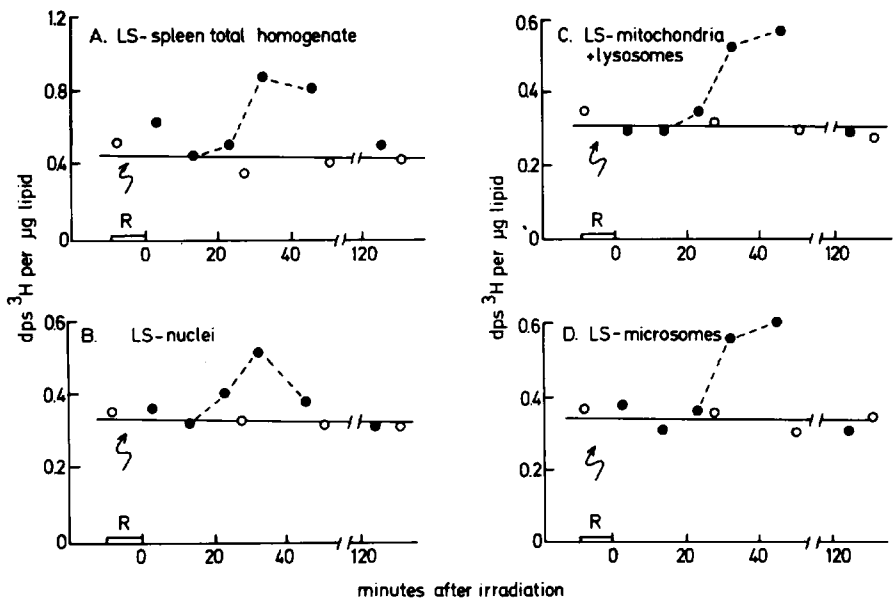

Fig. 4. Effect of X-irradiation on the incorporation of fatty acid label into the lipids of subcellular fractions of the lyphosarcoma (LS) cells. 5 Gy-irradiation was performed two hours after label injection.

nuclei (Fig. 5B). When comparing Fig. 5A and Fig. 5B an interesting phenomenon emerges. The appearance of the increase in free fatty acid label in the soluble fraction precedes the increased incorporation of label into the nuclear lipids. This again suggests that the radiation effects on intracellular phospholipid labelling may be the result of an enhanced availability of free fatty acids in the cytosol. The increase in the level of fatty acid label in the cells is not the result of an increased label concentration in the blood, because the specific activity of the lipids in the blood was found to remain constant (not shown).

The increased fatty acid concentration in the cytosol after irradiation conceivably is related to a transient increase in the rate of transport of the fatty acids over the plasma membranes. The difference in specific radioactivity between blood and cytosol will obviously be most pronounced shortly after the injection of the fatty acid label. Thus, the difference in specific radioactivity of the cyto-

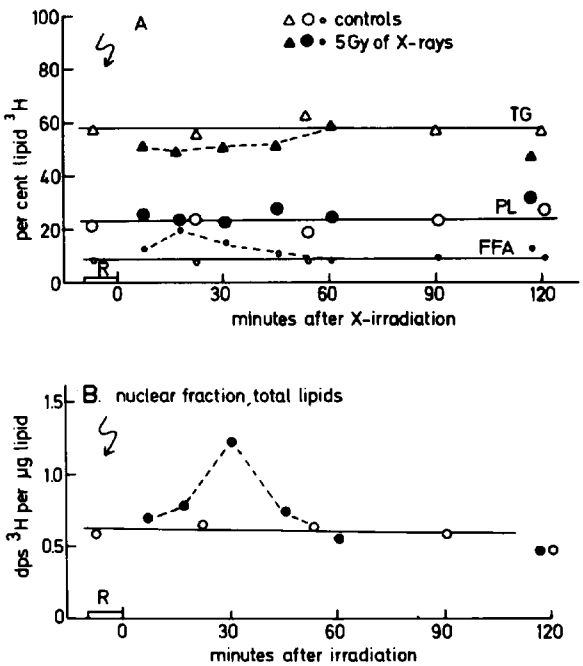

Fig. 5. The effect of X-irradiation on labelling of the free fatty acid pool in the soluble fraction of the tumor cells compared with that on the incorporation of fatty acid label into the nuclear lipids of the same cells. A Distribution of [^{3}H]label among the major lipid classes in the cytosol. B [^{3}H]-label incorporated in the nuclear lipids of the same tumor cells. The irradiation with 5 Gy of X-rays was performed two hours after label injection.

solic free fatty acids between irradiated and control cells will be most conspicuous when the animals are irradiated immediately after injection of [^{3}H]palmitate. This is demonstrated in Fig. 6 which presents an experiment in which animals were irradiated within 5 minutes after injection of the label. The radiation response in the lipids of the nuclear fraction is given in Fig. 6A. The effect of irradiation on the specific radioactivity of the free fatty acids in the cytosol is presented in Fig. 6B. The specific radioactivity of the free fatty acids is increased as a result of irradiation of the animals. These experiments suggest that incorporation of the fatty acids into the phospholipids of the tumor cells is enhanced because of an increase in the intracellular availability of fatty acids as a result of the irradiation.

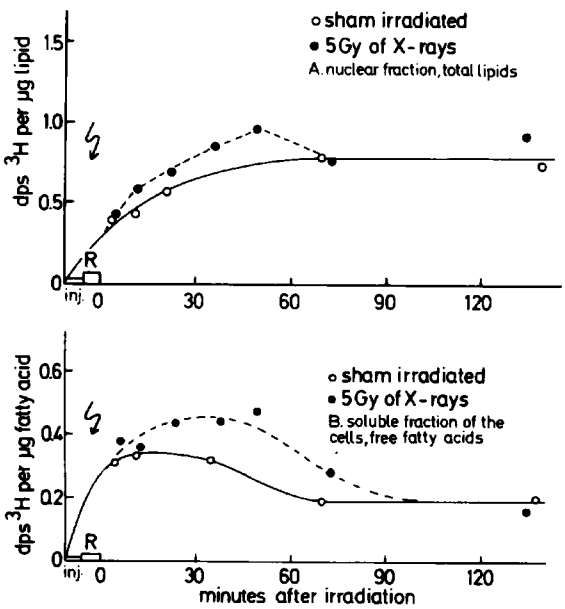

Fig. 6. Effect of 5 Gy of X-irradiation immediately after injection of tracer fatty acid.
A Incorporation of [^{3}H]label in nuclear acid. B Specific radioactivity of free fatty acids in the cytosol of the same cells Each mouse was injected intraperitoneally with 25 µCi of [^{3}H]-palmitate. Two mice were used for each point.

Irradiation of isolated tumor cells.

In order to substantiate our hypothesis that intracellular availability of fatty acid is the limiting factor in the incorporation of acyl groups into the tumor phospholipids, the following experiments were done. Isolated lymphosarcoma cells (10 ml) were incubated at 37°C with 0.5 ml of radioactive tracer fatty acids complexed to bovine serum albumin. The mixture was irradiated with 5 Gy of X-rays one hour after the start of the incubation.

A significant enhancement of specific radioactivity in the phospholipids of the cells was found at 30 and 45 minutes after irradiation (Table 2). As for the in vivo experiments, there was no difference in radiation effect between 16:0 and 20:4. Hypotonic treatment resulted in essentially the same pattern of specific radioactivity increase as irradiation, but the two effects were not additive. This suggests that the possible alteration in membrane structure caused by the hypotonic treatment may be of similar nature as that caused by irradiation. Either condition by itself leads to a transient increase in fatty acid flux, sufficient to attain a maximal level of acyl group incorporation into membrane lipids.

Table 2. The effect of X-irradiation and hypotonic shock on the specific radioactivity of the phospholipids of lymphosarcoma cells *in vitro*.

Conditions	Percentage above control value			
	Time after irradiation:			
	30 min		45 min	
	(16:0)	(20:4)	(16:0)	(20:4)
5 Gy of X-rays (R)	11 ± 2	9 ± 2	8 ± 2	9 ± 1
Hypotonic shock (H)	13 ± 1	12 ± 1	10 ± 2	14 ± 2
H + R	14 ± 3	10 ± 2	10 ± 2	13 ± 1

Data are given as percentage above the control value. The control had a specific activity in the phospholipids for ^{3}H (16:0) 10 dps per μg lipid and for ^{14}C (20:4) 0.35 dps per μg lipid. The hypotonic shock was given by two-fold dilution of the suspension with distilled water and incubating for 5 minutes at 37°C. Hereafter the cells were made isotonic and treated as indicated in the text and in the methods section.

DISCUSSION

From radiation studies on model systems performed in our laboratory, it is known that saturated acyl groups in phospholipids, such as palmitate, are more

radioresistant than the polyunsaturated acyl groups, such as arachidonate.[11] If X-irradiation would preferentially impair the polyunsaturated acyl groups in the cell membrane of lymphosarcoma cells, we would expect a more pronounced incorporation of arachidonate than of palmitate following X-radiation. This was not found. This could mean that the polyunsaturated fatty acyl groups in the phospholipids of the cellular membranes are effectively protected against the radiation injury. However, it is possible that the fatty acid incorporation is simply limited by the supply of fatty acids. If this is the case a preferential replacement of fatty acids can not take place because of a lack of substrate. The experiments described in this report strongly suggest that the latter holds true.

An enhanced fatty acid concentration in the cytosol became manifest immediately after or even during irradiation (with 5 Gy) and was instantly followed by an increased incorporation of fatty acid label in the phospholipids. The increased flux of fatty acids through the membrane points to a radiation-induced permeability increase for the fatty acids which lasts for no more than about 60 minutes. A second radiation treatment induces the same temporary change in permeability. Whatever the nature of the membrane disturbance is, apparently it is quickly restored.

We have preliminary indications that also in liver an increased fatty acid incorporation into the phospholipids takes place after irradiation. There is a suggestion in the literature[24] that fatty acid uptake might be rate-limiting for fatty acid metabolism in perfused heart. To the best of our knowledge no data are available for tumor tissues and for liver.

The observation that an enhanced specific radioactivity in the phospholipids could also be accomplished following irradiation of the tumor cells in vitro (Table 2), argues against the possibility that the in vivo results are influenced by indirect radiation effects. Also in the in vitro situation the radiation effects were the same for 16:0 and 20:4. Hypotonic treatment produced the same effect as irradiation and the two effects were not additive. Either treatment alone apparently is sufficient to cause maximal label incorporation. Again, the incorporation of 16:0 and 20:4 were the same. This indicates that the radiation-induced increase in incorporation does not reflect repair of radiation damage to unsaturated acyl groups. From the observations mentioned, we like to suggest that no extensive lipid peroxidation had taken place after the irradiation. This is in line with earlier experiments where no products of lipid peroxidation could be measured directly after the radiation.[13,23] It is conceivable that the radiosensitive polyunsaturated fatty acyl chains in the membrane phospholipids are effectively protected against extensive radiation induced lipid peroxidation by endogenous protective systems. Soluble SH groups and α-tocopherol are among the candidates for this protection.[11,13,22,23]

The entry of fatty acids through the plasma membrane as a result of the irradiation, in vivo, is of a transient nature. Preliminary results on the in vitro system indicate that the enhanced fatty acid entry is prolonged. More detailed experiments on isolated lymphosarcoma cells are published elsewhere.[25] As yet we can only speculate on the mechanism by which the transient increase in fatty acid flux through the membrane is accomplished. It has been reported[26,27] that radiation causes an increase in membrane lipid fluidity in erythrocytes. An increased membrane fluidity is known to enhance the permeability for small ions.[28] Likewise an increased membrane fluidity may affect the trans-membrane diffusion of free fatty acids. However, it is not firmly established by what mechanism fatty acids are transported over the membrane. Although passive diffusion has been suggested as the transport system,[29] a possible involvement of protein(s) in this process cannot be excluded.[30] Accordingly, the radiation effect we observe may also involve alterations at the membrane protein level.

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